



## Complement Activation-Related Cardiac Anaphylaxis in Pigs: Role of C5a Anaphylatoxin and Adenosine in Liposome-Induced Abnormalities in ECG and Heart Function

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**Abstract**

Cardiac anaphylaxis is a severe, life-threatening manifestation of acute hypersensitivity reactions to allergens and drugs. Earlier studies highlighted an amplifying effect of locally applied C5a on the process, however, the role of systemic complement (C) activation with C5a liberation in blood has not been explored to date. In the present study we used the porcine liposome-induced cardiopulmonary distress model for 1) characterizing and quantifying peripheral C activation-related cardiac dysfunction, 2) exploring the role of C5a in cardiac abnormalities and therapeutic potential of C blockage by sCR1 and an anti-C5a antibody (GS1), and 3) elucidating the role of adenosine and adenosine receptors in paradoxical bradycardia, one of the symptoms observed in this model. Pigs were injected i.v. with different liposomes (Doxil, MLV), zymosan, rhuC5a and adenosine, and the ensuing hemodynamic and cardiac changes (hypotension, tachy- or bradycardia, arrhythmias, ST-T changes, ventricular fibrillation and arrest) were quantified by ranking on an arbitrary scale (cardiac abnormality score, CAS). There was significant correlation between CAS and C5a production by liposomes in vitro, and the liposome-induced cardiac abnormalities were partially or fully reproduced with zymosan, rhuC5a, adenosine and the selective A1 agonist cyclopentyl-adenosine. The use of C non-activator liposomes or pretreatment of pigs with sCR1 or GS1 attenuated the abnormalities. The selective A1 blocker cyclopentyl-xanthine inhibited bradycardia without influencing hypotension, while the A2 blocker ZM24135 had no such effect. These data suggest that 1) systemic C activation can underlie cardiac anaphylaxis; 2) C5a plays a causal role in the reaction; 3) adenosine action via A1 receptors may explain paradoxical bradycardia, and 4) inhibition of C5a formation or action, or of A1 receptor function may alleviate the acute cardiotoxicity of liposomal drugs and other intravenous agents that activate C.

**Key Words:** adenosine, adenosine receptors, allergy, anaphylatoxins, anaphylactoid reactions, Bezold-Jarisch reflex , electrocardiography, cyclopentyl-xanthine, hemodynamic changes, hypersensitivity reactions, pseudoallergy

**Abbreviations:** C, complement; CAS, cardiac abnormality score; Chol, cholesterol; CO, cardiac output; CPA, cyclopentyl-adenosine; CPX, cyclopentyl xantine; DMPC, dimyristoyl phosphatidylcholine; DMPG, dimyristoyl phosphatidylglycerol; ECG, electrocardiography; GS1, an anti porcine C5a antibody; LUV, large unilamellar vesicles; MAP, mean arterial pressure; MLV, multilamellar vesicles; PA, arterial pressure amplitude; PAP, pulmonary arterial pressure; PBS, phosphate-buffered saline; pCO<sub>2</sub>, expiratory (end-tidal) CO<sub>2</sub>; rhuC5a, recombinant human C5a; SAP, systemic arterial pressure; sCR1, soluble C receptor type 1

## Introduction

Cardiac anaphylaxis is one of the most severe, potentially lethal manifestations of immediate allergy to a variety of allergens and drugs. It involves major abnormalities of cardiac electrical conductance and ventricular function, leading to heart rate changes with conductance blocks, arrhythmias, ventricular fibrillation and arrest, acute circulatory failure and, occasionally, death (6, 12, 30, 34, 51). As for its mechanism, activation of mast cells in the heart is a key underlying process (17, 18, 23, 24, 27, 28), although details of the reaction remain poorly understood. One of its controlling factors recognized in the past is the anaphylatoxin C5a, whose intracoronary administration was shown to amplify the electrical and mechanical response of isolated guinea pig heart to a variety of allergens (7, 8, 17, 18). However, in absence of information on the site

and amount of C5a formation during classical allergic reactions *in vivo* and the trafficking of anaphylatoxins into and within the heart, the physiological relevance of cardiac responses to locally applied C5a remains questionable. Another unsolved question in this area is whether C5a plays a role in the adverse cardiac effects of some complement (C) activating drugs, where prior exposure to the drug or to its components, and, hence, sensitization, cannot be established. Examples for the latter phenomenon include the acute cardiac disturbance caused by the anticancer drugs paclitaxel (Taxol<sup>®</sup>) (5, 19, 21, 32, 33, 50), liposomal amphotericin B (Ambisome<sup>®</sup>)(1), liposomal daunorubicin (DaunoXome<sup>®</sup>) (10) and liposomal doxorubicin (Doxil<sup>®</sup>) (2, 49). Taxol activates C via the emulsifier of paclitaxel, Cremophor EL (42, 48), while liposomal drugs do so via their phospholipid bilayer capsule (38, 41).

We reported previously that i.v. injection of a small amount of large multilamellar liposomes (MLV) (43, 47) or of Doxil (44) in pigs induces dramatic hemodynamic changes via C activation in peripheral blood. The clinical picture included cardiac abnormalities typical of anaphylaxis, suggesting that the model may have utility in the elucidation of the role of systemic C activation in this syndrome. Accordingly, the specific goals of the present study were 1) to characterize the cardiac electric and functional changes during liposome-induced peripheral C activation, 2) to develop criteria for quantitative assessment of cardiac abnormalities *in vivo*, 3) to explore the role of C5a in the changes and potential benefits of C5a blockade, and 4) to elucidate the roles of myocardial adenosine release and adenosine A1 and A2 receptors in paradoxical bradycardia, an intriguing phenomenon observed in our model that we report here for the first time.

## Materials and Methods

### Materials

*Chemicals and drugs.* Dimyristoyl phosphatidylcholine (DMPC), dimyristoyl phosphatidylglycerol (DMPG) and cholesterol (Chol) were purchased from Avanti Polar Lipids (Alabaster, AL). Zymosan, N<sup>6</sup>-cyclopentyl-adenosine (CPA), recombinant human C5a (rhuC5a) and adenosine were from Sigma Chem. Co. (St. Louis, MO). 4-(2-[7-Amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl)phenol (ZM 241385) was from Tocris (Ellisville, MO) and 8-cyclopentyl-1,3-dipropyl-3,7-dihydro-1-purine-2,6-dione (CPX) was generously provided by Dr. Ray Olsson, Department of Medicine, University of Florida, Tampa, FL. Recombinant soluble complement receptor type 1 (sCR1), murine anti-porcine C5a (GS1, Chemicon, Temecula, CA) and the antibodies for the pig C5a ELISA were provided by Avant Immunochemicals Inc. (Needham, MA), Dr. Gregory L Stahl (Boston, MA), and Professor Otto Goetze (Heidelberg, Germany) as detailed previously (45, 47).

*Liposomes.* The source, preparation and detailed characterization of Doxil, synthetic, saturated, large multilamellar and unilamellar vesicles (MLV and LUV) consisting of DMPC, DMPG and Chol (50:5:45 mole ratios) were described in previous studies in detail (43, 44, 47).

## **Experiments Using Pigs**

The Institutional Animal Care and Use Committee approved the procedures described, which was conducted in compliance with the Animal Welfare Act and adhered to the principles stated in the current Guide for the Care and Use of Laboratory Animals. Yorkshire swine in the 25-40 kg range were sedated with i.m. ketamine (500 mg), followed by intubation or tracheotomy, and anesthesia with isoflurane using a Narkomed 28 (North American Drager, Telford, PA) anesthesia machine. Inspired O<sub>2</sub> and end tidal CO<sub>2</sub> tensions were maintained at 21 vol % and 35-40 mm Hg, respectively. Continuous acquisition and analysis of ECG and hemodynamic data was done using a personal computer and in-house data acquisition and analysis software

“DataLyser” (developed in Labwindows CVI), compatible with a National Instruments (Austin, TX) data acquisition card. The data were acquired at 250 Hz sampling rate. ECG recording was started at 1-2 min before injection of test substances and lasted for 20 min post-injection. Heart rate was calculated from the R-R intervals, obtained from 10 consecutive QRST complexes on the ECG. Measurement of T wave amplitude and ST segment alterations were done by cursor operation. Other details of hemodynamic analysis, including equipment, placement of catheters and measurement of systemic and pulmonary arterial pressures (SAP and PAP), cardiac output (CO) and end-tidal PCO<sub>2</sub> were described previously (43, 47).

Liposomes, zymosan, C5a, adenosine and CPA were administered into the jugular vein as bolus injections (within about 5-10 sec), using phosphate buffered saline (PBS) as vehicle. Each injection was followed by 5-10 mL PBS wash. CPX and ZM24135 were administered in infusion, 10-15 min before the liposome injections. CPX was initially dissolved in ethanol which was then diluted 200-fold in saline. The C inhibitor sCR1 and GS1 were applied as described earlier (47).

### **Estimation of C5a Production by Liposomes In Vitro**

The C5a producing potency of 3 specified liposome preparations (MLV, Doxil and LUV) was assessed by incubation of 5 mg phospholipid/mL liposome with heparinized (20 IU/mL) pig plasma in vitro for 15 min at 37°C. The reaction was stopped by addition of 20 mM EDTA, followed by measurement of porcine C5a by ELISA. Details of the assay utilizing affinity purified porcine C5a, anti hog-C5a mAb T13/9, rabbit IgG against mouse IgG and biotinylated rabbit anti hog-C5a, was described earlier (14, 45). The calibration curve was linear in the 0.6-10 ng C5a/mL range.

## Statistical Methods

Data are presented as typical SAP and PAP curves, ECG tracings and means  $\pm$  S.D. for continuous variables. For analyzing the correlation between C5a formation in vitro and heart function in vivo, we developed a scoring system to provide a semiquantitative estimate of myocardial dysfunction on a scale of 1 to 5. The definition and details of cardiac abnormality scores (CAS) are described in the legend to Table 1. The correlation between CAS and C5a production by liposomes in vitro was analyzed by calculating the nonparametric (Pearson) correlation coefficient, which considers ranks without requiring normality in the distribution of values. All statistical analyses were done with GraphPad Prism.

## Results

### Characteristics of Liposome-Induced Cardiac and Hemodynamic Changes

Figs. 1-3 show typical cardiac and systemic hemodynamic changes that followed the injection of increasing doses of Doxil or MLV in pigs. They illustrate the common, as well as the differing features of more than a hundred reactions induced by Doxil, MLV and a variety of other reactogenic liposomes. The most prominent common feature of liposome reactions was their rapid development and reversibility, with most hemodynamic and ECG alterations returning to baseline, or near baseline, within 15-30 min. The variable features included the direction and extent of heart rate and SAP changes, the degree of pulmonary hypertension with or without reduction of CO and pCO<sub>2</sub>, and the nature and severity of arrhythmias. Thus, Fig. 1 demonstrates a moderate reaction to Doxil, characterized by an abrupt drop of mean arterial blood pressure (MAP) (panel A) that was associated with massive pulmonary hypertension, decreased CO and decreased pCO<sub>2</sub> (panel B). During the nadir of blood pressure curve, lasting

for about 4 minutes, we observed a transient tachyarrhythmic episode followed by ST depression and T wave elevation (Panel C, curves b-d, respectively). Although the MAP did not completely return to baseline, the ECG normalized after about 12-15 min (curve e). Fig. 2A presents a more severe reaction to (a higher dose) of Doxil, involving a deeper and longer hypotensive period compared to Fig. 1. This reaction was associated with severe bradycardia with arrhythmia, with the presence of incomplete as well as complete AV blocks with asystole (Fig 2B). Curves b-d in panel B of this figure show gradual increase of PQ interval leading to 2:1 AV block, suggesting that the bradycardia was not of sinus origin but rather a reflection of slowed AV conduction. Fig 3 illustrates a lethal reaction involving ventricular fibrillation and cardiac arrest within 3 minutes after the injection of MLV. Resuscitation of this animal with epinephrine is also documented as a sudden overshoot of MAP into the hypertension range.

Additional notable features of hypotensive liposome reactions included a greater reduction of systolic pressure compared to diastolic pressure, resulting in a substantial reduction of pulse pressure amplitude (Figs. 1-3). Furthermore, as illustrated in Fig 2, hypotension was often associated with bradycardia or bradyarrhythmia, although the physiological baroreflex response to hypotension is tachycardia. Hence, the phenomenon represents “relative”, or “paradoxical” bradycardia (9, 29). Quantification of the magnitude of MLV-induced bradycardia by averaging the increase of RR distances over baseline at maximal bradycardia gave  $278 \pm 31\%$  increases (mean  $\pm$  S.E.M, n= 7 pigs, 10 sec sampling times, MLV: 0.1 mg/kg). We also noted that the bradycardic and arrhythmic effects of liposomes showed significant positive correlation (linear regression analysis  $R^2: 0.51$ ,  $P \leq 0.005$ , n= 28 reactions) when maximal bradycardia (% of baseline) was plotted against the standard deviation of RR distances at the time of peak bradycardia (SD in the 0.05-0.5 sec range, 40 heart beats). This implies association between increased bradycardia and increased probability of arrhythmia, another indication of non-sinus

origin of bradycardia.

Fig. 4A shows an episode of major ST depression observed during severe reactions. When quantified and expressed as a function of time, the nadir of ST-depression appeared 4-6 minutes after the injection of liposomes, i.e., with 2-3 minutes delay relative to the peak of hemodynamic changes (Fig 4B and Figs 1C, curve C). A similar analysis of the time course of T wave elevation showed biphasic changes with peaks around 4-5 and 10-11 minutes (Fig 4C).

### **Quantification of Liposome-Induced Cardiac Dysfunction**

In light of the multitude and variability of liposome-induced cardiac abnormalities, it was impossible to use any of the measured ECG or hemodynamic parameters as a comprehensive index of cardiac dysfunction. We developed therefore a scoring system that took into consideration all cardiac electric and hemodynamic changes to differentiate between groups of symptoms with quantitatively distinguishable level of severity. Table 1 shows the key for this classification, based on the analysis of 111 liposome reactions in 63 experiments wherein pigs were injected with Doxil, MLV or other reactogenic liposomes. We differentiated 5 categories with increasing cardiac abnormality scores (CAS) in the 1-5 range, reflecting increasing severity from mild to lethal.

### **The Role of C5a in Liposome-Induced Cardiac Dysfunction**

*Correlation between in vitro C5a production and cardiac dysfunction in vivo.* To explore the role of C5a in the cardiac changes in our model, in one of three types of experiments we selected 3 liposome preparations that had substantially different in vivo reactogenicity, quantified the cardiac dysfunction they caused using CAS (Table 1), and correlated the CAS values with the C5a producing efficacy of these vesicles in pig serum in vitro. The 3 liposomes selected for

these studies were MLV, Doxil and LUV (c.f. Methods) that were previously reported to cause strong, intermediary and no hemodynamic side effects in pigs, respectively (43, 44, 47). In addition, we also used zymosan, a C activating yeast cell membrane extract, which too caused major hemodynamic changes in pigs (47). As shown in Table 2, C5a production by matched amounts of liposomes and zymosan showed significant correlation with CAS ( $P < 0.05$ ), supporting the notion that C5a plays a causal role in the cardiac abnormalities caused by these agents. In addition, the finding that the non-liposomal C activator zymosan also caused cardiac changes similar to those caused by high doses of Doxil or MLV provided evidence that the reactions were not due to a property unique to liposomes.

*Cardiac effects of C5a.* In the second series of experiments aimed at exploring the relationship between systemic C5a liberation and cardiac dysfunction, we injected pigs with increasing doses of rhuC5a using a dose range that was previously reported to cause hemodynamic abnormalities in various animals (15, 20). As shown in Fig. 5A, 330 ng/kg, which raised the baseline C5a level in pig blood (30-40 ng/mL (45)) by 30-40%, led to a mild (CAS 2) reaction with transient reduction of pulse pressure and slight, reversible hypertension. In sharp contrast, 440 µg/kg rhuC5a, which raised blood C5a by 600-800-fold, caused a short-lived transient hypertension followed by massive hypotension in association with bradyarrhythmia (Fig 5B,C), pulmonary hypertension (Fig 5C) and marked decrease of end tidal pCO<sub>2</sub> (Fig 5D). Thus, a large-dose of rhuC5a closely mimicked the severe cardiac abnormalities caused by strong C activator liposomes or zymosan.

*Cardiac effects of inhibitors of C5a formation and action.* Despite the robustness of the above relationships between cardiac abnormalities and C5a production and action, these data

presented only indirect evidence for a causal role of C5a in the reaction. To provide direct evidence, we revisited some unpublished data from our previous study analyzing the role of C activation in liposome-induced hemodynamic changes (47). This time we focused solely on the cardiac effects of sCR1, an inhibitor of C activation via the classical and alternative pathways, and those of an anti-porcine C5a antibody, GS1, which inhibits only the actions of porcine C5a (36). As reported earlier, the MLV-induced and C-mediated pulmonary changes were significantly inhibited by both agents (47). The present analysis indicated that in parallel with the reduction of pulmonary response to MLV, these inhibitors significantly reduced the cardiac abnormalities as well (Table 3).

### **Mechanism of Paradoxical Bradycardia**

In an effort to elucidate the mechanism of liposome-induced paradoxical bradycardia, we examined the heart rate and blood pressure responses of pigs following i.v. administration of liposomes, adenosine or the selective adenosine A1 receptor agonist, CPA, alone or in combination with CPX or ZM24135, drugs representing selective A1 and A2 receptor antagonists, respectively (11). Adenosine is known to cause bradycardia with peripheral hypotension (4, 26, 53), and, as shown in Fig 6A, these changes could be reproduced with an i.v. bolus of 0.3 mg/kg adenosine under our conditions. Fig. 6B shows that the bradycardic effect of exogenous adenosine was linear in the 0.1-0.6 mg/kg dose range. As shown in Table 4, adenosine, CPA, MLV, Doxil and zymosan all caused paradoxical bradycardia, i.e., decreased both the heart rate and MAP. CPX alone had a tachycardic effect and converted the bradycardic effect of all above reaction triggers into considerable tachycardia. However, importantly, the hypotensive effects of these agents were not, or were minimally inhibited by CPX, implying selective inhibition of bradycardia. ZM24135, the selective A2 blocker had no such effects as

CPX, suggesting that bradycardia was mediated primarily by cardiac adenosine A1 receptors. Taken together, these data suggest that acute adenosine release within the heart could explain paradoxical bradycardia via A1 receptors.

## Discussion

### **Cardiac Anaphylaxis and the Role of Complement**

Cardiac anaphylaxis, part of an acute and complex multi-system reaction, is the most severe manifestation of hypersensitivity to a variety of allergens including food, pollens, venoms and, importantly, certain drugs. Its pathomechanism involves activation of cardiac mast cells in the coronary arterial intima and perivascularly, in close proximity to myocytes (22-24). As for the role of C, cardiac mast cells express high affinity receptors for C3a and C5a whose triggering by anaphylatoxins induces the release of a variety of inflammatory mediators and vasoactive molecules (22-24). Thus, C5a was shown to intensify the allergen-induced anaphylactic crisis in isolated perfused guinea pig hearts (7, 18) leading del Balzo et al. to suggest that C activation functions as an amplification system in cardiac anaphylaxis (7, 18). However, the physiological relevance of the latter information is not clear without evidence that allergen-induced C activation, which is usually mild and occurs at the site of allergen exposure (52), leads to reactogenic levels of C5a in the heart despite the extremely short (seconds to minutes) half-lives of anaphylatoxins (15, 20). This lack of crucial information regarding the “C amplification of cardiac anaphylaxis” theory, together with the unexplained adverse cardiac effects of C activating liposomes and other drugs (see below) prompted the present study to examine whether peripheral C activation can explain cardiac anaphylaxis *in vivo* in a large animal model.

### **Pathophysiology of C activation-Related ECG Changes**

As previously outlined in detail, liposome-induced and C-mediated hemodynamic changes in pigs result from multiple interdependent adverse processes, including eicosanoid (mainly thromboxane A2) mediated pulmonary and coronary vasoconstriction (43, 47) possibly combined with microthrombus formation and microembolization of capillaries by neutrophil-platelet aggregates (25, 35, 37, 47). The resultant falls in left cardiac preload and coronary flow lead to myocardial ischemia, decreased contractility, reduced cardiac output and hypotension, all feeding a vicious cycle that either resolves spontaneously, or leads to death via circulatory collapse. The present study further refines this scheme inasmuch as we suggest that ischemic adenosine release from the heart causes bradycardia with arrhythmias, and, hence, it represents an additional factor aggravating cardiac dysfunction. Accordingly, the ECG changes -catalogued and analyzed for the first time in the present study-, can most easily be rationalized with ischemia-related membrane dysfunction plus adenosine-induced and A1-mediated electric conduction problems, as discussed below in more detail.

Although cardiac Purkinje fibers and the working cardiomyocytes are not known to express G-protein-linked receptors that mediate ion channel conduction changes in response to anaphylatoxins (16, 31), the late (after 10-15 min) ECG alterations, particularly the protracted and biphasic T wave changes, could reflect direct membrane damage in the cells caused by the terminal C complex (C5b-9) (13, 37).

### **Adenosine Release as Underlying Cause of Paradoxical Bradycardia**

One of the original observations in this study was the occurrence of a special physiological phenomenon referred to as relative, or paradoxical bradycardia (9, 29). Our focus on adenosine as underlying cause of this event was based on the facts that even moderate myocardial ischemia can lead to substantial local adenosine production and release from the heart, and that extracellular adenosine is known to cause bradycardia via hyperpolarization of Purkinje fibers in the sinus and AV nodes, and throughout the entire cardiac conduction system (4, 26, 53). Also, del Balzo et al described that the negative dromotropic effect of intra-coronary injection of rhuC5a in isolated guinea pig hearts was mediated by adenosine (8).

Our findings that adenosine and CPA mimicked, while CPX inhibited the bradycardic effect of i.v. C activators corroborate the above data of del Balzo et al. (8) and extend their observations inasmuch as we provide pharmacological evidence for a critical role of A1 receptors in the phenomenon. In addition, our experiments with CPX provide arguments against another possible explanation of paradoxical bradycardia: operation of the classical vagus-mediated Bezold-Jarisch reflex that may underlie vasovagal syncope and sudden death (3). The phenomenon involves nonspecific irritation of cardiac chemo- or stretch-sensitive mechanoreceptors leading to arterial hypotension, bradycardia and apnea. There are two major reasons why our experiments argue against the involvement of the Bezold-Jarisch reflex in C activation-related paradoxical bradycardia. First, CPX is not known to inhibit cholinergic neurotransmission (11), and second, CPX led to pharmacological uncoupling of bradycardia from arterial hypotension, which is not easy to reconcile with the joint, central inhibition of sympathetic vasoconstrictor and cardio-accelerator and respiratory centers in the medulla oblongata during the Bezold-Jarisch reflex (3). Nevertheless, in the absence of specific experimental evidence or literature data it cannot be *a priori* excluded that adenosine, via A1

receptors, may trigger a selectively heart-slowing cardiopulmonary chemoreflex, or that it selectively interferes with the cardiac cholinergic system in pigs (in fact, CPX itself caused tachycardia, Table 4). Exploration of these possible mechanisms was beyond the scope of this investigation and may require further studies.

### **The Causal Role of C5a in Liposome-Reactions**

The correlation between (i) liposome-induced C5a formation in pig serum in vitro and the degree of adverse reactions in vivo, (ii) the reproduction of cardiac anaphylaxis with rhuC5a and (iii) the inhibition of ECG abnormalities with inhibitors of C5a formation or action provide strong support for a key causal role of C5a in liposome-induced cardiac changes in our model. The experiments using rhuC5a also allowed for some calculations with regard to the extent of C5a rise during mild and severe cardiac reactions to exogenous C5a. Thus, considering that the normal plasma levels of C5 and C5a in pigs are approximately 175 µg/mL and 20 ng/mL, respectively, and that the plasma volume in pigs is ~33 mL/kg, the barely reactogenic 330 ng/kg rhuC5a dose led to about 30-40% rise of plasma C5a, while the 440 µg/kg rhuC5a dose, which mimicked severe cardiac anaphylaxis, caused ~600-700-fold rise of C5a. Thus, it is possible that severe liposome reactions may involve several hundred-fold rise of plasma C5a in pigs. It should be added though that in the absence of intermediary test doses and information on the clearance rate of rhuC5a in pig blood, it is difficult to apply these figures to humans. Also, our study did not rule out a significant role for C3a in the most severe or lethal reactions.

### **The Clinical Relevance of Complement-Mediated Cardiac Anaphylaxis**

The clinical relevance of our study lies in highlighting the possible mechanism by which Taxol, Doxil and some other C activating liposomal or micellar drugs and radiocontrast media

(39, 40) can cause acute cardiac adverse events. In the case of Taxol, cardiac arrests was a major obstacle in the development of this drug (32, 33) and fatalities can still occur despite mandatory anti-allergic premedication of patients (5, 19, 21, 50). With Doxil, the box insert warns of the presence of “acute infusion-related reactions in up to 10% of patients” (49), with “adverse cardiac events possibly or probably related to Doxil®“ (i.e., not to Doxorubicin) in 4.3% of patients (2). Among the symptoms tachycardia, bundle branch block, ventricular arrhythmias and heart arrest are listed (49). It should be emphasized with Doxil, however, that a major thrust of its clinical application vis-à-vis free Doxorubicin is the reduction of long term cardiotoxicity, which in fact is achieved with liposome encapsulation (49). Considering that the above anticancer drugs are used in the treatment of some half million new cases of lung, breast and ovarian cancer each year in the USA, the occurrence of C-mediated cardiac events can be roughly estimated in the order of hundreds to thousands per year, indicating a serious but preventable public health issue.

### **Concluding Remarks**

In extending our previous reports on the unique cardiopulmonary response of pigs to i.v. liposomes (43, 44, 46, 47) the present study quantified the cardiac changes and showed that these changes can be caused, at least in part, by C5a. Complement activation could represent an independent pathogenic pathway, a yet unclassified subgroup of cardiac anaphylaxis whose occurrence, and, hence, clinical significance may far outweigh classical cardiac anaphylaxis. Inhibitors of C activation or C5a or adenosine action might be useful in preventing or ameliorating “C activation-related cardiac anaphylaxis”. As further “spin-off”, our model may allow non-invasive and non-pharmacologic triggering of transient myocardial ischemias in anesthetized closed-chest pigs, which may provide a potentially useful new tool in studying the

mechanism and prevention of acute ischemic events in the heart.

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## FIGURE LEGENDS

**Figure 1.** Hemodynamic and ECG changes in a pig following bolus injection of Doxil. A) Real-time tracing of SAP curve. Doxil was administered into the jugular vein at 0.1 mg lipid/kg. B) Time course of the changes of PAP, end-tidal pCO<sub>2</sub> and CO during the same reaction. C) ECG tracings taken at the time points shown in Panel A.

**Figure 2.** Similar experiment as shown in Fig. 1, except that the dose of Doxil was doubled.

**Figure 3.** Hemodynamic and ECG changes in pigs following bolus injection of (0.5 mg/kg) MLV. The circles show the time of ECG recordings. Typical experiment illustrating a lethal ventricular fibrillation leading to cardiac arrest. E, administration of 0.1 mg/kg epinephrine (E).

**Figure 4.** A) Illustration of massive ST depression during a severe liposome reaction caused by MLV (0.5 mg lipid/kg). B) Progression of ST depression and C) progression of T wave elevation following injection of MLV (0.1 mg/kg) in pigs. The data in B and C represent % of baseline, means  $\pm$  SD in 6-9 pigs wherein ST depression and T wave elevation were measured at the indicated times.

**Figure 5.** Cardiopulmonary and ECG changes caused by injection of recombinant human C5a. A) Injection of 330 ng/kg rhuC5a. B-D) Injection of 440  $\mu$ g/kg rhC5a. B, SAP; C, PAP; D, pCO<sub>2</sub>. The animal was resuscitated with epinephrine (panel B). Typical experiment out of 3 independent tests.

**Figure 6.** A) Blood pressure and ECG changes in a pig following i.v. bolus of 0.3 mg/kg adenosine. B) Dose dependence of the bradycardic effect of adenosine. Mean  $\pm$  SD in 4 pigs.

**TABLE 1. Quantification of liposome-induced cardiac abnormalities in pigs.**

<b>ECG Abnormalities</b>	<b>Hemodynamic and Cardiorespiratory Alterations</b>	<b>Qualitative description</b>	<b>CAS<sup>1</sup></b>
Arrhythmia episodes, transient tachycardia	No or minimal changes in PA, SAP, CO, PAP and pCO <sub>2</sub> .	minimal	1
Longer lasting arrhythmia with tachycardia	Moderate rise of SAP and reduction of PA, no or minimal changes in CO, PAP and pCO <sub>2</sub>	mild	2
Major arrhythmias with tachycardia and/or bradycardia	Initial rise followed by moderate declines in SAP, PA, CO and pCO <sub>2</sub> , moderate rise of PAP	moderate	3
Major arrhythmias with tachycardia and/or bradycardia, ST depression/T wave changes	Dramatic and extended declines in SAP, PA, CO and pCO <sub>2</sub> , major rise of PAP	severe	4
Major arrhythmias with tachycardia and/or bradycardia, ST depression/T wave changes, cardiac arrest with or without ventricular fibrillation	Dramatic, extended and irreversible declines in SAP, PA, CO and pCO <sub>2</sub> , maximal rise of PAP. Fatal without CPR <sup>2</sup>	lethal	5

Abbreviation used only in this Table: CPR, cardiopulmonary resuscitation; PA, arterial pressure amplitude. <sup>1</sup>CAS, cardiac abnormality score, an arbitrary rank based on the severity ECG (column 1) and associated hemodynamic and cardiorespiratory abnormalities (column 2).

<sup>2</sup>Administration of i.v. epinephrine (0.01-0.1 mg/kg) with or without chest compression and/or electroconversion. The grouping of symptoms was based on the analysis of 111 liposome reactions triggered in a total of 63 pigs over several years of experimentation with the model.

Each CAS category listed was observed at least 22 times.

**TABLE 2 Association between C5a production and ECG changes caused by liposomes in pigs.**

Inoculum	C5a (% of baseline) <sup>a</sup>			CAS <sup>b</sup>		
	Mean	SD	n	Mean	SD	n
PBS	0	0	3	0	0	58
LUV	106	19	3	1.2	0.4	5
Doxil	371	35	3	3.3	0.8	15
MLV	608	16	3	4.1	0.7	20

<sup>a</sup>ELISA readout:  $A_{410}$  at 15 min/ $A_{410}$  at 0 min  $\times 100 \pm SD$  of triplicate determinations in a pig's serum that displayed typical reactivity to liposomes. <sup>b</sup>Cardiac abnormality scores, defined in Table 1, were determined in each pig following the first injection of matched amounts of liposomes. The Pearson  $r$  for the correlation between the mean C5a and mean CAS values is 0.978,  $P= 0.0219$  (two-tailed),  $R^2=0.957$ .

**TABLE 3. Inhibition of large multilamellar liposome-induced cardiac changes in pigs by blockers of complement activation and action.**

Treatment <sup>a</sup> mg/kg	Inhibition of reaction (% decrease of PAP) (n) <sup>b</sup>	Cardiac Abnormality Score (CAS) <sup>c</sup>	
		before	after
sCR1, 0.2	28, 71 (2)	4, 4 (2)	2, 1 (2)
sCR1, 2.0	40, 99 (2)	4, 3 (2)	2, 1 (2)
GS1, 1.6	41.3±12.5 (4)	3.8±0.5 (4)	2.6±0.6 (4)

The pulmonary hypertensive responses to 5 mg MLV (0.10-0.16 mg lipid/kg) was measured in pigs before and after treatment of the animals i.v. with the specified doses of soluble C receptor type 1 (sCR1), and GS1, a porcine anti-C5a antibody (see Methods and ref. 43). <sup>b</sup>The post-treatment rise of PAP was normalized for the pretreatment rise to quantify the drugs' inhibitory effect on pulmonary hypertension in terms of percentage. Entries are % inhibition obtained by the formula: 100-(PAP<sub>post</sub>/PAP<sub>pre</sub>) where PAP<sub>post</sub> and PAP<sub>pre</sub> mean the MLV-induced rise of PAP before and after drug treatment. Individual values with n=2 or mean ± SD for n=4 animals. These data were reported earlier (47). CAS values for the reactions before and after treatment (before, after). The "after" values are significantly lower than the "before" values for GS1, and the post-treatment values are also consistently lower for the 2 doses of sCR1, with 2 animals in each group.

**TABLE 4 Predominant role of adenosine and A1 receptors in mediating complement activation-related paradoxical bradycardia.**

Reaction Trigger/ inoculum	Endpoints					
	Heart Rate, % of baseline			MAP, % of baseline		
	Treatment					
Control	CPX	ZM241385	Control	CPX	ZM241385	
PBS	100	128 ± 12 (6)	100	100	100	100
Adenosine	52 ± 3 (9)	123 ± 12 (5)	61 ± 7 (10)	62 ± 2 (9)	71±10(5)	74 ± 4 (10)
CPA	46 ±6 (3)	93 ± 4 (4)	-	66±5(3)	83 (1)	-
MLV	73±4 (13)	111 ± 4 (15)	73 ± 11 (2)	42 (2)	59±12(3)	48 ± 1 (2)
Doxil	86±2 (4)	118±11 (6)	-	65+/-6 (4)	64±8 (6)	-
Zymosan	58 ± 8 (6)	169 ± 10 (5)	-	51±8(6)	59±7(3)	-

Values are means ± SE for (n) bolus i.v. injections in up to 9 pigs. Entries are heart rate and MAP readings at the peak of reactions, expressed as % of pre-injection baseline. 100 % implies no significant change. The doses of reaction inducers and inhibitors were: adenosine, 0.6 mg/kg; CPA, 0.3 mg/kg; CPX, 0.25 mg/kg; ZM241385, 1.5 mg/kg; MLV and zymosan, 0.2 mg/kg; Doxil, 0.01 mg/kg (doxorubicin). In the case of Doxil, because of tachyphylactic responses, only the first injections were considered. Abbreviations only in this table: HR, mean regular heart rate, -, not tested

Fig. 1

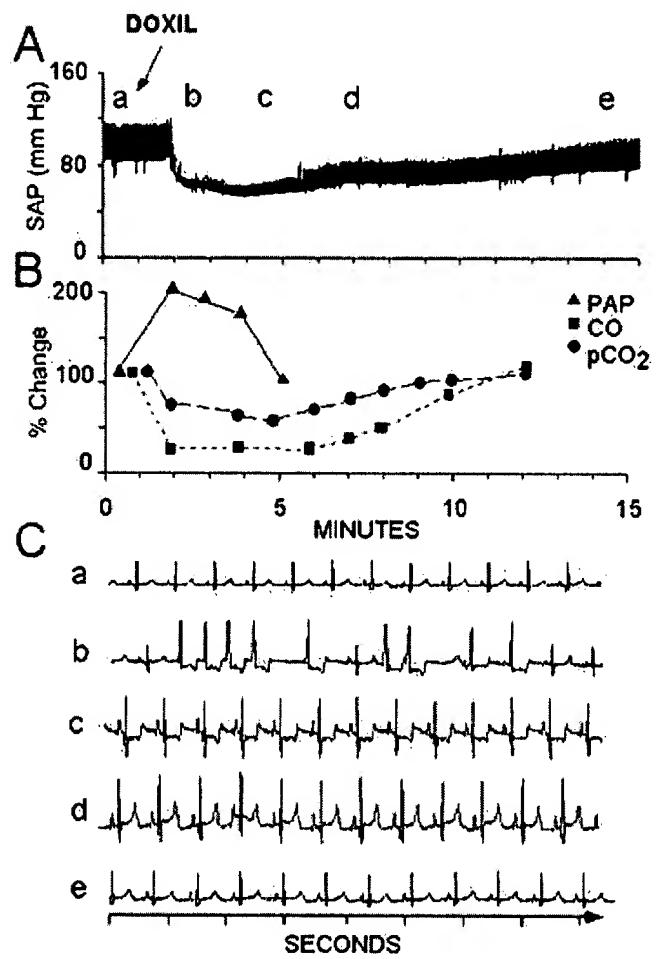


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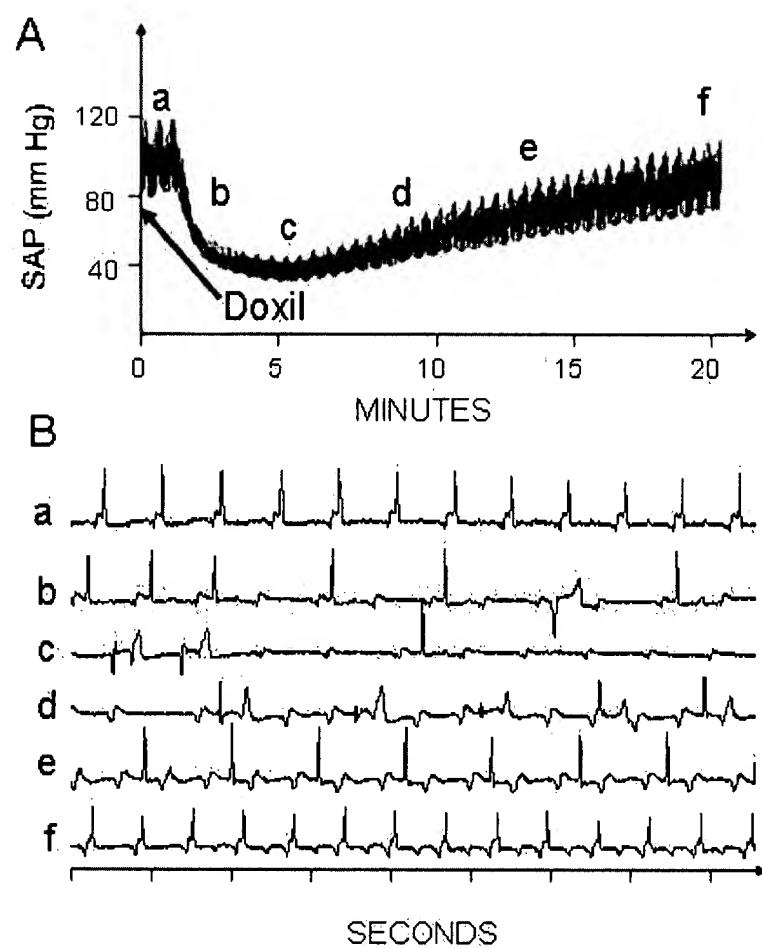


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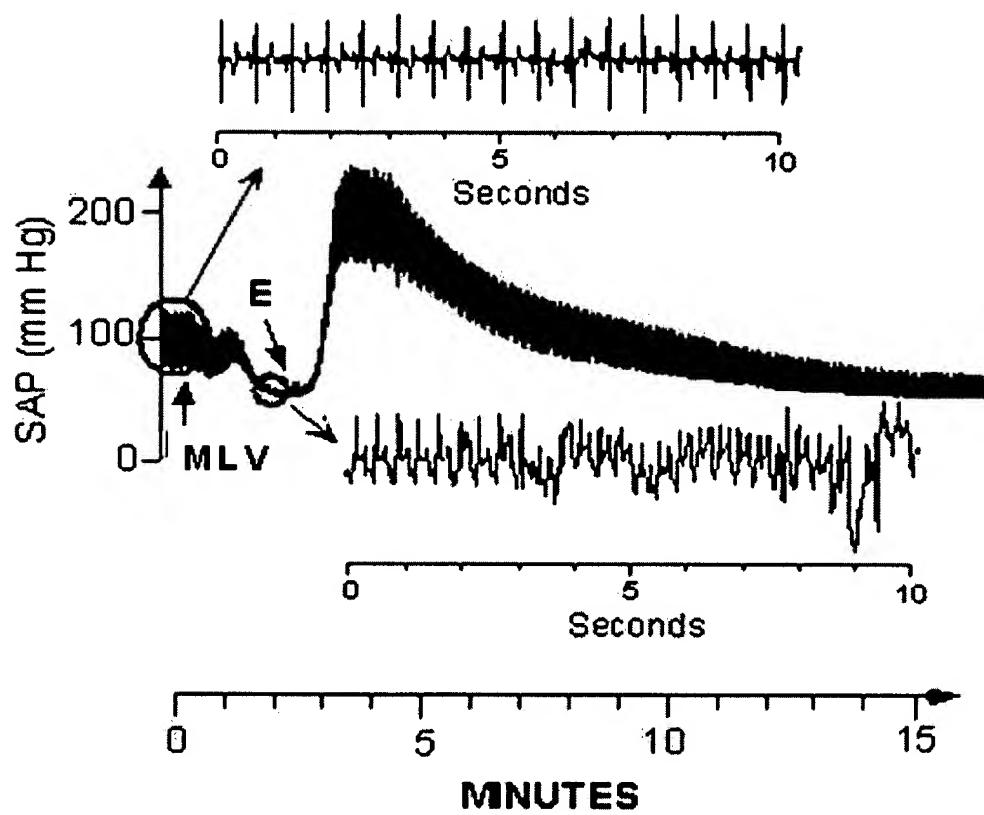


Fig. 4

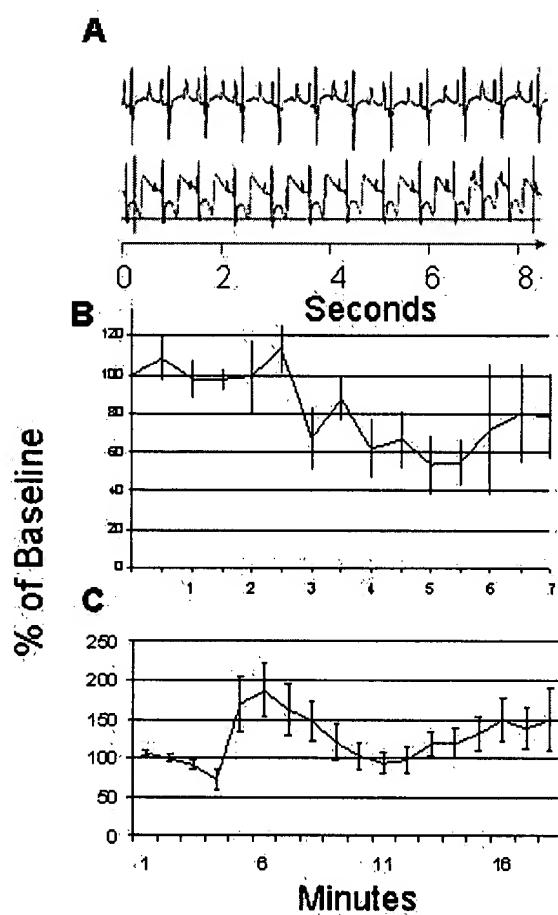


Fig. 5

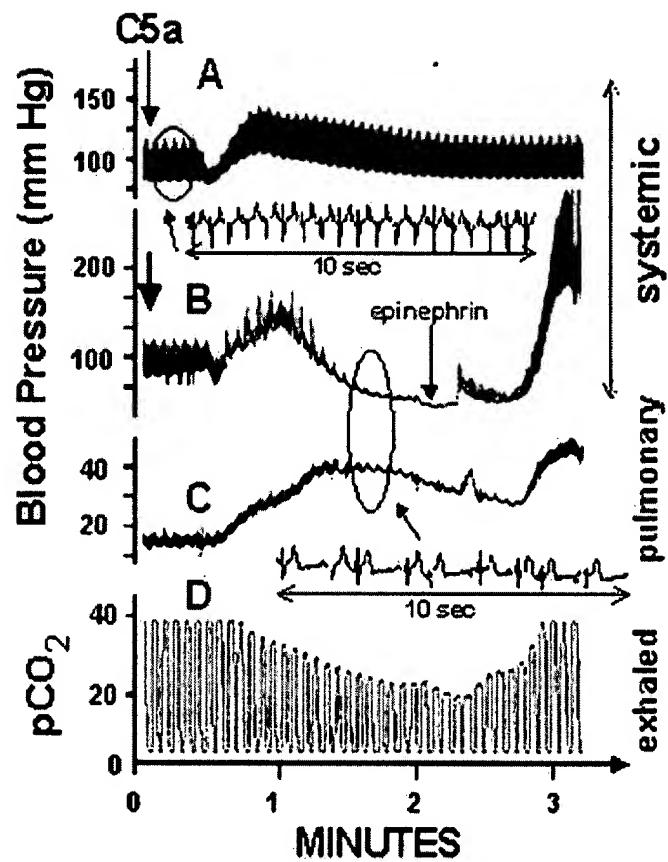
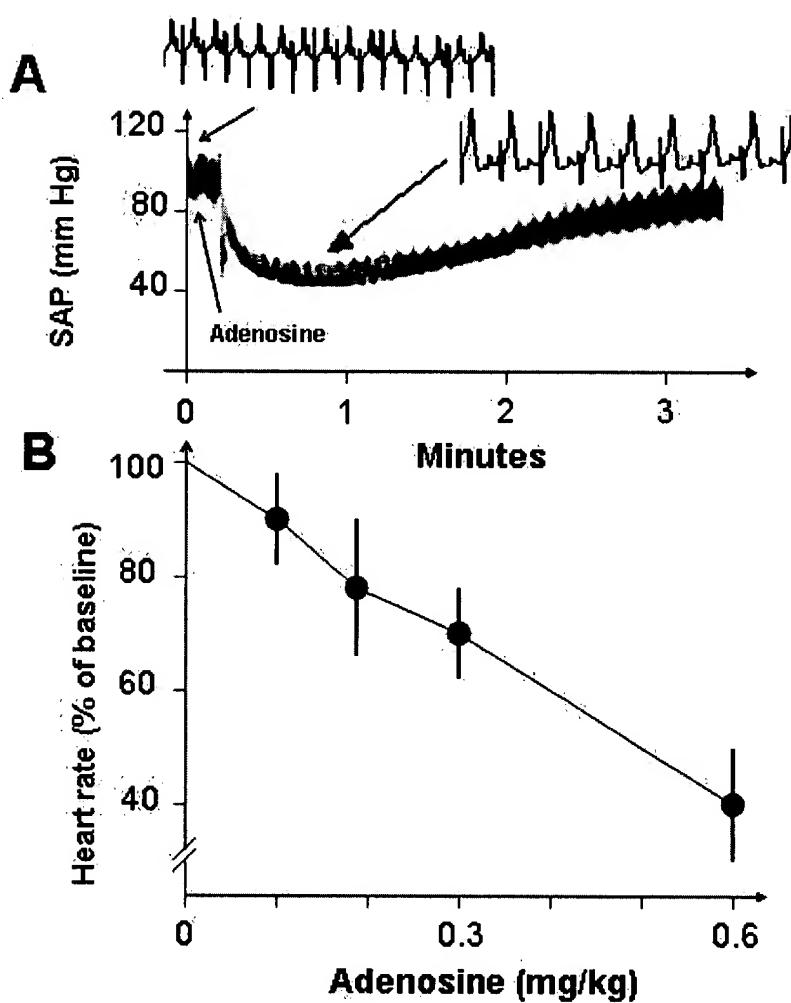


Fig. 6



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## CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

# ANTI-C5A MONOCLONAL ANTIBODY REDUCES CARDIOPULMONARY BYPASS AND CARDIOPLEGIA-INDUCED CORONARY ENDOTHELIAL DYSFUNCTION

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 Accepted for publication Aug 2, 1998. Address for reprints: Frank W. Sellke, MD, Division of Cardiothoracic Surgery Beth Israel-Deaconess Medical Center, East Campus, Dana 905, 330 Brookline Ave, Boston, MA 02215.

**Objective:** Because C5a induces tissue injury by activating polymorphonuclear leukocytes, the hypothesis was that inhibition of C5a activity would reduce cardioplegia-related injury.

**Methods:** Pigs were placed on cardiopulmonary bypass. The hearts were arrested for 1 hour with hyperkalemic cardioplegia. Pigs were then separated from bypass, and the hearts were reperfused for 2 hours. Anti-porcine C5a monoclonal antibody (1.6 mg/kg, intravenously; n = 6) was administered 20 minutes before the onset of cardiopulmonary bypass. Six pigs received saline solution vehicle. Reactivity of coronary arterioles was studied in vitro with videomicroscopy. Microvessels from uninstrumented pigs served as controls for vascular studies.

**Results:** Endothelium-dependent relaxation to adenosine diphosphate (percent relaxation of precontraction) was reduced after cardioplegic reperfusion ( $63\% \pm 14\%$  vs  $77\% \pm 10\%$  in

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control at 10 µmol/L;  $P = .01$ ). This impairment in endothelium-dependent relaxation was improved with anti-porcine C5a monoclonal antibody ( $80\% \pm 22\%$ ;  $P = .01$  vs saline solution), as was the impaired endothelium-dependent relaxation to clonidine ( $64\% \pm 12\%$  control;  $26\% \pm 17\%$  saline solution;  $55\% \pm 24\%$  anti-porcine C5a monoclonal antibody at 10 µmol/L;  $P = .01$  saline solution vs control or anti-porcine C5a monoclonal antibody). Myeloperoxidase activity was significantly decreased ( $0.2 \pm 0.2$  units/g protein;  $P = .04$ ) in the anti-porcine C5a monoclonal antibody group compared with  $5.2 \pm 2.7$  in the saline solution group.  $\text{CH}_{50}$  2 hours after bypass was not statistically different ( $0.57 \pm 0.41$  unit and  $0.65 \pm 0.41$  unit, respectively) between the anti-porcine C5a monoclonal antibody and saline solution groups. Despite less myocardial polymorphonuclear leukocyte infiltration after C5a inhibition, maximum rate of rise of left ventricular pressure, percent segmental shortening, and blood flow through the left anterior descending coronary artery were similar in the anti-porcine C5a monoclonal antibody and saline solution groups.

**Conclusions:** Inhibition of C5a limits neutrophil-mediated impairment of endothelium-dependent relaxation after cardiopulmonary bypass and cardioplegic reperfusion, but it has no effect on short-term myocardial functional preservation.

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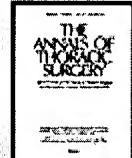
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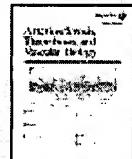
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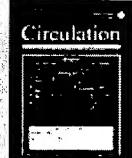
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**Hemodynamic Changes Induced by Liposomes and Liposome-Encapsulated Hemoglobin in Pigs : A Model for Pseudoallergic Cardiopulmonary Reactions to Liposomes: Role of Complement and Inhibition by Soluble CR1 and Anti-C5a Antibody**

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